

Naval Medical Research Institute

Bethesda, MD 20889-5607

NMRI 93-38

June 1993



2
HJ

AD-A266 834

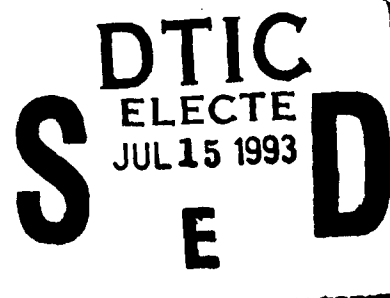


AN APPARATUS FOR MEASURING THE BIOLOGICAL OXIDATION
OF HYDROGEN GAS UNDER HYPERBARIC CONDITIONS

S. R. Kayar
G. S. Goehring
W. E. Long, Jr.
A. L. Harabin

Naval Medical Research
and Development Command
Bethesda, Maryland 20889-5606

Department of the Navy
Naval Medical Command
Washington, DC 20372-5210



Approved for public release;
distribution is unlimited

93 07 8

93-16010



NOTICES

The opinions and assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the naval service at large.

When U. S. Government drawings, specifications, or other data are used for any purpose other than a definitely related Government procurement operation, the Government thereby incurs no responsibility nor any obligation whatsoever, and the fact that the Government may have formulated, furnished or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

Please do not request copies of this report from the Naval Medical Research Institute. Additional copies may be purchased from:

National Technical Information Service
5285 Port Royal Road
Springfield, Virginia 22161

Federal Government agencies and their contractors registered with the Defense Technical Information Center should direct requests for copies of this report to:

Defense Technical Information Center
Cameron Station
Alexandria, Virginia 22304-6145

TECHNICAL REVIEW AND APPROVAL

NMRI 93-38

The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed by the NMRI scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

ROBERT G. WALTER
CAPT, DC, USN
Commanding Officer
Naval Medical Research Institute

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED			1b. RESTRICTIVE MARKINGS	
2a. SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for Public Release; distribution is unlimited.	
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE				
4. PERFORMING ORGANIZATION REPORT NUMBER(S) NMRI 93-38			5. MONITORING ORGANIZATION REPORT NUMBER(S)	
5a. NAME OF PERFORMING ORGANIZATION Naval Medical Research Inst.		6b. OFFICE SYMBOL (if applicable)	7a. NAME OF MONITORING ORGANIZATION Bureau of Medicine and Surgery	
5c. ADDRESS (City, State, and ZIP Code) 2301 Wisconsin Avenue Bethesda, Maryland 20889-5607			7b. ADDRESS (City, State, and ZIP Code) Department of the Navy Washington, DC 20372-5120	
5d. NAME OF FUNDING/SPONSORING Naval Medical Research and Development Command		6b. OFFICE SYMBOL (if applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
5c. ADDRESS (City, State, and ZIP Code) 8901 Wisconsin Avenue Bethesda, Maryland 20889-5605			10. SOURCE OF FUNDING NUMBERS	
			PROGRAM ELEMENT NO. 61153N	PROJECT NO. MRO4101
			TASK NO. OOD-1103	WORK UNIT ACCESSION NO. DN241522
11. TITLE (Include Security Classification) (U) AN APPARATUS FOR MEASURING THE BIOLOGICAL OXIDATION OF HYDROGEN GAS UNDER HYPERBARIC CONDITIONS				
12. PERSONAL AUTHOR(S) S.R. Kayar, G.S. Goehring, W.E. Long Jr., and A.L. Harabin				
13a. TYPE OF REPORT TECHNICAL REPORT		13b. TIME COVERED FROM 01/91 TO 02/93	14. DATE OF REPORT (Year, Month, Day) 1993 June	15. PAGE COUNT 33
16. SUPPLEMENTARY NOTATION				
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP	SUB-GROUP		
			Hydrogen diving, hydrogen, metabolism, diving	
19. ABSTRACT (Continue on reverse if necessary and identify by block number)				
<p>We designed, constructed, and operated an apparatus to permit us to expose mammalian tissues to high pressures and concentrations of hydrogen (H_2), using the radioisotope tritium (T_2) as a label. As part of a study of H_2 as a breathing gas for deep diving, we needed to determine if mammalian tissues have a latent capacity to metabolize H_2 that is only evident under hyperbaric conditions. The apparatus was designed to store up to 1 Curie T_2 between experiments, mix T_2 and H_2 with air in the experimental chamber without producing an explosive mixture, and reclaim the T_2 at the end of the experiment for safe disposal. The T_2 was purchased in glass ampules. Storage of T_2 was accomplished by placing the T_2 ampule in a steel pressure chamber and breaking the ampule to release the T_2 under complete containment. The potential problems from explosivity were solved by diluting the T_2 and H_2 with helium. Reclamation of the T_2 was accomplished by passing a controlled stream of the T_2 and H_2 along with a controlled stream of air through a combustion trap. The combustion trap was filled with</p>				
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input checked="" type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT <input type="checkbox"/> DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL Pegina E. Hunt, Command Editor			22b. TELEPHONE (Include Area Code) (301) 295-0198	22c. OFFICE SYMBOL MRL/RSP/NMRI

palladium-coated molecular sieve material, which catalyzed the reaction of O_2 with T_2 and H_2 to form water. The T_2 was thus safely disposed of by discarding the combustion trap in the radioactive solid waste.

Results from exposing mammalian tissue dice, homogenates, or live cell cultures to 10 or 50 atm H_2 and 8 mCi T_2 indicated that the tissues were incorporating quantities of the label only on the order of 10^{-8} Ci \cdot ml $^{-1}$. This amount was indistinguishable from that found in control samples of saline or distilled water exposed along with the tissues. Positive controls of palladium beads and cultures of the H_2 -metabolizing bacterium *Alcaligenes eutrophus* incorporated over 1000 times more of the tritium label. We concluded that mammalian tissues do not oxidize H_2 under hyperbaric conditions, with a limit of detectability of 10^{-7} mol $H_2 \cdot g^{-1} \cdot min^{-1}$.

Accession For	
NTIS CRA&I	<input checked="" type="checkbox"/>
DTIC TAB	<input type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification	
By	
Distribution /	
Availability Codes	
Dist	Avail and/or Special
A-1	

DTIC QUALITY INSPECTED 8

TABLE OF CONTENTS

ABSTRACT	i
ACKNOWLEDGEMENTS	iv
INTRODUCTION	1
MATERIALS AND METHODS	2
General Description of Apparatus	2
Safety Features	3
Chamber Function and Experimental Design	7
RESULTS	10
DISCUSSION	11
Biological Significance	11
Isotope Effects and Contaminants	12
CONCLUSION	14
REFERENCES	15
APPENDIX	17
TABLE 1	20
FIGURE LEGENDS	21
FIGURE 1	23
FIGURE 2a	24
FIGURE 2b	25
FIGURE 3	26
FIGURE 4	27
FIGURE 5	28
FIGURE 6	29

ACKNOWLEDGEMENTS

We are most grateful to Dr. Daniel Arp of Oregon State University, Dr. Paul Lamburger of Mound Laboratory, Miamisburg, Ohio, and Dr. Göte Östlund and Mr. Richard Oleson of the University of Miami for their insights into the use of tritium. We thank Dr. Susan Pocotte, Dr. Thomas McKenna, and Dr. Douglas Robinson for their generous gifts of cell cultures.

This work was supported by the Naval Medical Research and Development Command Work Unit No. 61153N MR04101.00D-1103. The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the U.S. Navy or the naval service at large.

INTRODUCTION

Hydrogen (H_2) is currently being evaluated as a major component of a breathing gas for deep diving. We must determine if mammalian tissues have the capacity to oxidize H_2 when subjected to high H_2 pressure and concentration. It has long been known that H_2 is biologically inert in mammals when they are exposed to low concentrations at a pressure of 1 atmosphere (Séguin and Lavoisier, 1789). A study with the radioactive hydrogen isotope tritium (T_2) further confirmed this observation (Smith et al., 1953).

Gaseous H_2 is an extremely stable molecule that does not spontaneously dissociate into ions at atmospheric temperature and pressure (Farkas, 1935). However, numerous bacteria have enzyme systems that catalyze this reaction as part of a major metabolic pathway (Smith and Marshall, 1952). Hydrogen metabolism has also been reported in some algae, flagellate protozoans, and plants (Adams et al., 1981). In many of these organisms, the direction of the biochemical reactions in the pathway is reversible, either forming or consuming H_2 , depending on the concentration of H_2 (Schlegel, 1989).

Hydrogen has been used as an experimental diving gas in some studies with humans and other animal species (Edel et al., 1972; Örnham, 1987; Cosson, 1990). At pressures over 20 atm H_2 , narcotic and even psychotic effects (Cosson, 1990) have been uncovered that are inconsistent with the assumption that H_2 is biologically inert in mammals. It is thus essential to test for an oxidation of H_2 under hyperbaric conditions of high H_2 concentration to determine if mammalian tissues have a latent capacity to oxidize H_2 that is not expressed under normobaric conditions of low H_2 concentration.

In this report we describe the design, operation, and first use of an apparatus that permitted us to expose isolated mammalian tissues to H_2 under high pressure, using T_2 as a tracer. Oxidation of H_2 by the tissues was assayed by scintillation counting to determine if tritium had been incorporated in the tissue samples. The apparatus permitted reclamation of the unused T_2 gas as tritiated water, thus assuring safe and convenient disposal of the radioactive tracer.

MATERIALS AND METHODS

General Description of Apparatus

A simplified diagram and flow chart of the tritium apparatus appears in Fig. 1. The tissues to be studied were placed in dishes that were stacked inside a pressure chamber. The T_2 was stored in another pressure chamber and diluted in helium (He). To begin an experiment, a small quantity of T_2 was released into the experimental chamber. As the T_2 passed through the system, it flowed through a chamber containing a water vapor absorbent (anhydrous $CaSO_4$, Drierite®, WA Hammond Drierite Co., Xenia, OH); this removed tritiated water vapor, which was found to be a serious contaminant of the T_2 . The amount of T_2 drawn for the experiment (7.6 - 8.0 mCi) was computed from the known dilution of the T_2 in its storage chamber, the measured volume of the free gas space in the experimental chamber, and the pressure increase in the experimental chamber when the T_2 was added.

The experimental chamber was pressurized with He to 10 atm and then with H_2 to a total gauge pressure of either 20 or 60 atm (10 or 50 atm H_2). The tissues remained for 1 h under pressure.

To decompress, the chamber gases were vented through a flow meter. A stream of air passed through another flow meter, and the two gas streams combined. This combined gas stream passed through a combustion trap filled with palladium-coated molecular sieve material. The palladium catalyzed the combustion of H_2 and T_2 with O_2 to produce water, which was then absorbed by the molecular sieve material. All the tritiated water produced from the experiment was expected to be absorbed by one combustion trap. However, a second combustion trap linked in series with the first one served to assure that no T_2 escaped the system. A H_2 sensor was used on the line leading to the building exhaust and was set to ring if any H_2 or T_2 were being vented. Because the combination of H_2 with O_2 liberates heat, temperature sensors were placed at several critical locations in the exhaust system: at the junction between the air and chamber gas streams, and on each of the two combustion traps. The system was considered to be operating safely when the sensor on the junction showed no elevation over room temperature, and both combustion traps were below $50^\circ C$.

Safety Features

The most important constraints placed on apparatus design and operation were those dealing with the explosive nature of many concentrations of H_2 and O_2 mixtures, the high diffusivity of H_2 and T_2 , and the radioactivity of T_2 . A detailed diagram of the apparatus, including all chambers, valves, and pressure gauges appears in Fig. 2.

The entire apparatus was placed in a high-flow hood that vented over $1000\text{ ft}^3 \cdot \text{min}^{-1}$ ($28,000\text{ l} \cdot \text{min}^{-1}$). The hood was located in a room that was specially designed for working with radioactive and explosive gases. The room was at negative pressure

relative to the rest of the building, in order to vent any accidental leaks to the outside. Air flow through the room was rated at over $1500 \text{ ft}^3 \cdot \text{min}^{-1}$ ($42,000 \text{ l} \cdot \text{min}^{-1}$).

The T_2 (Dupont - New England Nuclear, Boston, MA) was purchased as 1 Ci (0.38 ml) in a glass ampule. After extensive searching of commercial distributors for radiotracers, we found that T_2 was available only in quantities of 1 Ci or more. A full Curie is far too much to use for a single experiment; such a quantity would be expected to induce tissue damage and massive radioisotope effects. Thus, the T_2 containment portion of the apparatus had to be designed to allow us to purchase 1 Ci but use mCi quantities per experiment and safely store the remainder for later experiments.

The chamber (PC1) used to store the T_2 was leak-tested with He for 2 weeks. After the T_2 ampule was placed inside PC1 but before the ampule was broken, PC1 was pressurized to 300 psi with He and observed for 2 days to check again for leaks. Once the T_2 ampule was broken inside PC1, the containment case that housed PC1 was an additional safety measure against leaks. Gauge G5 mounted on this case allowed us to check for an increase in pressure in the containment case as an indication of a leak from PC1; such a leak would not be detected as quickly by a drop in pressure in the chamber on G1.

The brass chambers (PC2 and PC3) were initially leak-tested with H_2 before the entire apparatus was assembled. Chamber PC3 was pressurized to 300 psi with H_2 . After 1 month, the pressure had not dropped. Chamber PC2, which was of identical design to PC3, was leak-tested for 1 week with H_2 . All piping in the manifold system was also leak-tested with H_2 at the pressures to be used in experiments.

The non-explosivity limits for H_2 and O_2 mixtures are from 0 - 5% and from 95 - 100% H_2 in O_2 (Eichert and Fischer, 1986). When chamber PC3 was first sealed with material inside for an experiment, the chamber contained air with an O_2 concentration of 21%. The T_2 could be added directly to this chamber safely because the T_2 was highly diluted in He to far less than the flammability limit. However, the H_2 could not be added until the O_2 concentration had been reduced to 5% or less. This problem was resolved by compressing the chamber first with He to 10 atm, making the O_2 concentration fall to 2%. The H_2 could then be added to the system to any desired final pressure, without creating an explosive mixture.

The design of the exhaust side of the system had to take into consideration that the chamber gases were explosive if in uncontrolled contact with air, and that 8 mCi radioactivity could not be routinely released from the building. The combustion traps (Fig. 2b) and their manifolds solved both of these problems. The relative flows of the chamber gas and air had to be maintained at the prescribed flow ratio of 1/100 in order to remain well within the safety limits for mixtures of H_2 and O_2 . The manifolds and flow meters for the air and chamber gas streams were designed to be of volumes differing by approximately 100-fold, and special markings on the flow meters assisted in maintaining this flow ratio. However it was ultimately the operator's obligation to adjust the two gas flows and monitor the flow meters continuously during chamber decompression. Given these constraints, decompression rate in practice was 0.4-0.5 atm \cdot min⁻¹.

A thermistor (Cole-Parmer) was used to monitor the temperature of the pipe at the junction point of the chamber gas and air streams, to check for any rapid increases in

temperature. Thermistors were also attached to the two combustion traps to verify that they were not heating past their limit of 50 °C, as recommended by the manufacturer (Science Glass, Inc.). Operating procedure mandated monitoring these three thermistors continuously during decompression, and decreasing or shutting off chamber gas flow if a problem arose.

The amount of water per experiment produced in the combustion traps could be estimated from the number of moles of H_2 and T_2 used (24.2 g H_2 at 22 °C = 1 mole H_2 which oxidizes to 1 mole H_2O , weighing 18 g). The combustion traps were capable of retaining up to 8 g water with >99.9% efficiency (Östlund and Mason, 1974). They could retain up to 12 g at a lower efficiency, as demonstrated by measuring a weight increase of 12 g in the first trap, and also a weight increase of a few grams in the second trap in series with it. Thus, the two traps were both a safety measure to assure 100% efficiency of T_2 removal, and an economy measure to derive maximal use of each trap. When the first trap was calculated to be full, it was removed and weighed. The second trap was also weighed and shifted to the position of the first trap, and a new trap was installed in the second position. The full trap was discarded in the solid radioactive waste. Thus, the greatest part of the radioactive waste from these experiments was stored in glass containers, from which beta energy cannot escape, and absorbed into molecular sieve material. If a full trap were ever dropped and broken, the molecular sieve material could be swept up and discarded without contaminating the building.

The H_2 sensor that was located downstream of the combustion traps was used to verify that all T_2 and H_2 had been eliminated from the gas stream. The operator was also obligated to monitor this sensor continuously during decompression. The H_2 sensor

was set to ring a loud alarm if it detected over 2% H_2 , i.e. over 50% of the flammability limit of 4% H_2 . Under normal chamber decompression, the H_2 sensor registered between 0 and 1% flammability (0 - 0.04% H_2), which was not different from room air.

Chamber Function and Experimental Design

Calibration

To determine the activity of T_2 supplied per experiment, it was necessary first to compute the free gas volume of the experimental chamber (PC3). Volume displacement with water demonstrated that the total volume of the empty chamber was 250 ml. However, the experimental material, which consisted of fluids, plastic dishes, and a dish holder, occupied some of this space. The manifold above V8 was designed to fit into a Luer lock on a syringe. After loading the chamber with the experimental material and sealing it, the valves leading to the experimental chamber were opened. A gas-tight syringe was mounted and a known volume of air was injected into the system. Valve V8 was quickly closed and the pressure increase in the chamber was recorded. Free gas volume in the chamber was computed from the relationship $P_1/V_1 = P_2/(V_1 + V_i)$, where P_1 is the initial pressure in the chamber (1 atm), V_1 is the gas volume of the chamber to be solved for, P_2 is the new pressure in atmospheres after injecting air, and V_i is the volume of air injected. In a typical experiment, V_i was found to be 210 ml.

The activity of T_2 supplied per experiment was computed from the free gas volume in the experimental chamber, the dilution of T_2 in its carrier He, and the pressure increase in the experimental chamber when the T_2 was added. For example, 1 Ci T_2 in the 330 ml storage chamber at 300 psig (21.4 ATA) gives a concentration of 7.06 ml gas per mCi. To deliver 8 mCi T_2 to an experiment requires 56.5 ml of gas. Adding 56.5 ml

to a chamber with a free gas volume of 210 ml will increase the pressure from 1 atm to 1.27 atm, or 4 psig.

The activity of T_2 routinely used in these experiments was 7.6 - 8.0 mCi. This amount was selected as one that should be easily detected if it were incorporated. A much higher activity could have caused major tissue damage from irradiation, whereas a much lower activity would have been difficult to deliver accurately with the size and pressure limits of our apparatus.

Mammalian Tissue Experiments

Organs and cell cultures used in this study, taken from guinea pigs, rats, and pigs, are listed in Figs. 3 and 4. Two positive control experiments, one using an organic substance, and one using an inorganic substance, were also performed to test that tritium activity would be found in samples in which H_2 oxidation was known to occur.

Alcaligenes eutrophus is a gram negative bacterium that is known to metabolize H_2 (Probst et al., 1979); cultures of this bacterium were tested as the organic positive control. Palladium catalyzes the oxidation of H_2 with O_2 to form water; Pd beads immersed in water were used as the inorganic positive control. Sterile, distilled water was used as a negative control.

The tissue samples and controls (3 ml each) were placed in sterile petri dishes that had been prepared with small notches in their rims to ensure gas entry into the dishes when lidded and stacked. The seven petri dishes for each experiment were stacked in an aluminum holder and placed inside the experimental chamber. The middle dish of each stack contained saline as a control. Two additional dishes containing desiccant were used for further removal of tritiated water vapor contamination. One of these desiccant

dishes, which was perforated along its edges and bottom for easy gas penetration, sat on top of the aluminum holder directly at the site of gas entry into the chamber. The second desiccant dish was at the top of the stack of tissue dishes, and was not lidded.

After introducing the T_2 into the chamber, it was allowed to mix with the air in the chamber for 1 - 2 min. Failure to allow mixing led to a marked stratification of the tritium label remaining in the experimental dishes post-dive. Compression rate with He and H_2 was $2 \text{ atm} \cdot \text{min}^{-1}$, following a standard human dive protocol, to ensure viability of the cells used in the experiments. Post-dive cell viability was confirmed in the live cultures by microscopic examination. Survivorship was comparable to that under lab bench conditions over the same time period; over 95% for the PC12 and endothelial cells, and 10-30% for the cardiac myocytes.

Following the 1-hour exposure to T_2 and the chamber decompression, samples of $20 \mu\text{l}$ were taken from each of the petri dishes and added to vials containing 10 ml of "scintillation cocktail" (Atomlight, Dupont-New England Nuclear, Boston, MA). The vials were shaken vigorously by a vortex mixer for 2 min, with intermittent stopping and uncapping of the vial, to facilitate elimination of residual T_2 gas from the samples. The activity of tritium remaining in the samples was measured using a liquid scintillation counter (Wallac 1410, Pharmacia, Gaithersburg, MD). Minimal additional loss of tritium activity in the samples over the next few days indicated that most of the T_2 gas had indeed been eliminated.

RESULTS

Quantities of tritium on the order of 10^{-12} moles $T_2 \cdot g^{-1}$ of tissues, or 10^{-9} Curies $\cdot ml^{-1}$ of saline were found in the mammalian tissues studied, at both 10 and 50 atm H_2 (Figs. 3 and 4). At the relative dilutions of T_2 in H_2 we used, this would imply an uptake of 1.5×10^{-8} moles $H_2 \cdot g^{-1}$ tissues $\cdot min^{-1}$. To compute the time period for this rate, we included both the 1-hour exposure at constant pressure and the time during decompression (0.5 h from 10 atm, and 2 h from 50 atm) when the tissues were exposed to the same dilution of T_2 in H_2 as at constant pressure (Table 1).

However, a similar quantity of the tritium label was also present in the saline and distilled water control dishes. Analysis of variance indicated that there were no significant differences in tritium activity between the various tissue dishes and the saline control dish in each experiment ($p = 0.11$, $F = 1.86$, $df = 6$ at 10 atm, and $p = 0.29$, $F = 1.26$, $df = 6$ at 50 atm). There was 2 to 3-fold variability between experiments in the tritium background found in the saline control dishes. The tritium activity in the mammalian tissue experiments was normalized for this variation in background by subtracting the activity found in the saline control dish in each experiment from the activity in each of the tissue dishes. Analysis of variance of the normalized data indicated that there were no significant differences between the different tissues ($p = 0.13$, $F = 1.58$, $df = 14$ at 10 atm, and $p = 0.24$, $F = 1.32$, $df = 14$ at 50 atm), or between any of the tissues and the distilled water analyzed as its own experiment ($p = 0.18$, $F = 1.43$, $df = 15$ at 10 atm, and $p = 0.14$, $F = 1.51$, $df = 15$ at 50 atm).

The positive controls of Pd immersed in water and the suspensions of *A. eutrophus* showed several orders of magnitude more tritium activity than the mammalian tissues

(Fig. 5 and 6). This activity was in the range of 10^{-5} Ci \cdot ml $^{-1}$ for both positive controls at 10 and 50 atm H₂. Corresponding values in mass-specific units were on the order of 10^{-7} moles T₂ \cdot g $^{-1}$ bacteria and 10^{-9} moles T₂ \cdot g $^{-1}$ Pd. This implies an uptake of H₂ of 1.2×10^{-3} moles H₂ \cdot g $^{-1}$ bacteria \cdot min $^{-1}$ and 1.3×10^{-5} moles H₂ \cdot g $^{-1}$ Pd \cdot min $^{-1}$ for the 1-hour exposure at constant pressure plus decompression times from 10 and 50 atm H₂ (Table 1).

DISCUSSION

Biological Significance

The picomole quantities of tritium found in the samples of mammalian tissues were not different from those found in the saline and distilled water controls. We therefore concluded that the mammalian tissues have no detectable capacity to oxidize H₂ under hyperbaric conditions of high H₂ concentration, at a limit of detectability of 10^{-7} moles H₂ \cdot g $^{-1}$ tissues \cdot min $^{-1}$.

The high tritium activity in the positive controls indicated that the apparatus and experimental protocol were adequate to demonstrate H₂ oxidation. In the case of the bacterial suspensions, our estimated H₂ oxidation rates (Table 1) are very close to the rate of 1.6×10^{-3} mol H₂ \cdot g $^{-1}$ \cdot min $^{-1}$ calculated by spectrophotometry in suspensions of *A. eutrophus* bubbled with H₂ under 1 atm conditions (unpublished data).

The 8 mCi of T₂ to which we exposed all the mammalian tissues and controls had a solubility in water of 10.7 pMol \cdot ml $^{-1}$ (685 nCi \cdot ml $^{-1}$). A true background for tritium with our scintillation counter and cocktail was only roughly 0.23 pCi (50 disintegrations per minute per vial of scintillation cocktail). Thus, the quantity of tritium label found in

the mammalian tissues and water controls (Figs. 3 and 4) was very low compared to the quantity of T_2 used, but higher than a background in the absence of direct exposure to T_2 . A major aspect of the use of this apparatus and the interpretation of the data we obtained with it was an examination of this unexpectedly high experimental background.

Isotope Effects and Contaminants

Smith et al. (1953) exposed tissues of rats to T_2 at low concentrations and under 1-atm conditions. The activity of tritium that they found incorporated is in the same order of magnitude as that found in the mammalian tissues in this study, after correcting for differences in the amount of T_2 used. Smith et al. (1953) attributed this trace incorporation to the formation of tritiated water by what they termed "physical exchange reactions", and not to an oxidation of H_2 gas by mammalian cells.

MilliCurie quantities of T_2 are known to produce radioactive isotope effects that leave deceptively high background counts (Smith et al., 1953; G. Östlund, personal communication). An exchange between hydrogen atoms in H_2 gas molecules and hydrogen atoms in water molecules is energetically unfavorable at room temperature and pressure (Farkas, 1935). However, the radioactivity of the T_2 evidently provides the energy needed for an isotope exchange between tritium atoms in T_2 gas molecules and ordinary hydrogen atoms in water molecules. Wilzbach (1957) described this isotope exchange phenomenon between T_2 and a variety of organic molecules.

In addition, tritiated water vapor may be a contaminant in commercially supplied T_2 , despite manufacturers' analyses to the contrary (Arp, 1989; P. Lamburger, personal communication). Indeed, we had considerable difficulty initially in reducing the background to the level we have reported; this required passing the T_2 through or over a

desiccant three times before it reached the dishes containing tissues or saline. The desiccant trap through which the T_2 passed was found to pick up at least 20% of the T_2 activity that we calculated we used in the experiments. The two dishes of desiccant sitting in the top of the experimental chamber were found to pick up a few tenths of a percent more of the T_2 activity. Before the installation of these desiccants, our background for tritium label in water controls was higher than the solubility of T_2 in water, at the partial pressure of T_2 gas we calculated we were using. This observation was what led us to conclude that the T_2 was contaminated with a tritium-labeled molecule of high water solubility; tritiated water vapor (THO) was the most likely candidate. We requested an analysis of the T_2 from the manufacturer. The mass spectrometric analysis showed that the T_2 was 97.6152 moles % pure, and THO was not listed as a trace contaminant. However, discussions with several researchers who are experts on tritium maintained that they find evidence of THO in their T_2 routinely (D. Arp and P. Lamburger, personal communication), and that the presence of water vapor by mass spectrometry is unusually difficult to demonstrate (P. Lamburger, personal communication).

Tritiated water vapor was found to be extremely volatile, whenever it was known to have been produced in experiments. In the positive control experiments (Figs. 5 and 6), the saline control dish was found to contain roughly 10 times more tritium activity than the saline control dish in each of the mammalian tissue experiments. We attribute this to tritiated water being formed in the dishes with Pd or bacteria, and then evaporating and condensing into the saline dish. When such an experiment was left sealed for several days, all the dishes came into equilibrium with each other in their tritium activity.

Despite the drawbacks of working with a radioisotope, the use of T_2 as a tool had the advantage of extreme sensitivity; we had good reproducibility in measuring the tritium label in 10^{-12} mole quantities (Figs. 3 and 4), which would not have been possible with H_2 electrodes. Deuterium (D_2) could potentially have been used for these experiments and would not have caused the isotope exchange problems. However the use of D_2 has its own limitations; it must be measured by mass spectrometry, which would not have been as sensitive as liquid scintillation counting of T_2 .

CONCLUSION

The apparatus that we have designed and built for testing the capacity of mammalian tissues to oxidize H_2 under hyperbaric conditions was successful. Tritiated water vapor entering the system with the T_2 or produced during the experiment was a serious problem; this contaminant made it difficult to distinguish low incorporation rates of T_2 from background. The mammalian tissues and live cell cultures that we tested did not incorporate any more of the tritium label than that amount found in sterile water or saline exposed under identical conditions. The bacterial culture studied as a positive control had a rate of H_2 oxidation estimated from T_2 incorporation that was very close to the rate estimated under 1 atm conditions using standard biochemical techniques. We concluded that mammalian tissues have no detectable capacity to oxidize H_2 under hyperbaric conditions of high H_2 concentration.

REFERENCES

- Adams, M. W. W., L. E. Mortenson, and J. S. Chen. Hydrogenase. Biochimica et Biophysica Acta, Vol. 594, pp. 105-176, 1981.
- Arp, D. J. Hydrogen-oxidizing bacteria: Methods used in their investigation. In: Modern Methods of Plant Analysis New Series, Vol. 9, Gases in Plant and Microbial Cells, edited by H. F. Linskens and J. F. Jackson. Berlin: Springer-Verlag, 1989, pp. 257-274.
- Cosson, P. Le syndrome hydrogène: Aspects séméiologiques, neuro-physiologiques, neuro-chimiques et neuro-pharmacologiques évoquant une participation étiologique dopaminergique. Centre d'Études et de Recherches Biophysiques Appliquées à la Marine, Report # 90-03, 1990, pp. 76.
- Edel, P. O., J. M. Holland, C. L. Fischer and W. B. Fife. Preliminary studies of hydrogen-oxygen breathing mixtures for deep sea diving. In: The Working Diver. Symposium Proceedings, Columbus, OH, Feb. 22-23, 1972, pp. 257-270.
- Eichert, H. and M. Fischer. Combustion-related safety aspects of hydrogen in energy applications. International Journal of Hydrogen Energy, Vol. 11, pp. 117-124, 1986.
- Farkas, A. Orthohydrogen, parahydrogen and heavy hydrogen. London: Cambridge University Press, 1935, pp. 186-191.
- Örnham, H. Swedish animal experiments using hydrogen-oxygen mixtures at pressures up to 6.0 MPa. In: Hydrogen as a Diving Gas, edited by R.W. Brauer. Proceedings of the 33rd Undersea and Hyperbaric Medical Society Workshop, 1987, pp. 25-28.

- Östlund, H. G. and A. S. Mason. Atmospheric HT and HTO. I. Experimental procedures and tropospheric data 1968-72. Tellus, Vol. 26, pp. 91-102, 1974.
- Probst, I., G. Wolf and H. G. Schlegel. An oxygen-binding flavohemoprotein from *Alcaligenes eutrophus*. Biochimica et Biophysica Acta, Vol. 576, pp. 471-478, 1979.
- Schlegel, H. G. Aerobic Hydrogen-oxidizing (Knallgas) bacteria. In: Autotrophic Bacteria, edited by H.G. Schlegel and B. Bowien. Berlin: Springer-Verlag, 1989, pp. 305-329.
- Séguin, A. and A. Lavoisier. Premier mémoire sur la respiration des animaux. Mémoires de l'Académie des Sciences (Paris), pp. 566-584, 1789.
- Smith, G. N., R. J. Emerson, L. A. Temple, and T. W. Galbraith. The oxidation of molecular tritium in mammals. Archives of Biochemistry and Biophysics, Vol. 46, pp. 22-30, 1953.
- Smith, G. N. and R. O. Marshall. Fixation of molecular tritium by bacterial suspensions. Archives of Biochemistry and Biophysics, Vol. 39, pp. 395-405, 1952.
- Wilzbach, K. E. Tritium-labeling by exposure of organic compounds to tritium gas. Journal of the American Chemical Society, Vol. 79, pp. 1013, 1957.

APPENDIX

Operating Procedure for Apparatus

The construction diagram for the T_2 apparatus appears in Figs. 2a and 2b. A stainless steel pressure chamber (PC1; 330 ml internal volume) with a 0 - 600 psi pressure gauge (G1; US Gauge, Ametek, Baltimore, MD) was used to hold the T_2 gas. The T_2 was purchased as 1 Ci (0.38 ml) in a glass ampule (Dupont - New England Nuclear, Boston, MA). The ampule was placed inside the stainless steel chamber, along with three small steel balls. The chamber was sealed, flushed, and then pressurized with He to 300 psi. The chamber was vigorously shaken to break the ampule and release the T_2 into the carrier He. Valve V1 was used initially to control the gas flow of this chamber. After the ampule was broken, the chamber was mounted into a plexiglass containment case and connected by stainless steel piping to the rest of the apparatus. Valve V1 was opened, and the flow from the chamber was controlled by valves V2 and V3. The containment case was then sealed. Pressure in this case was monitored with a 0-30 psi pressure gauge (G5; US Gauge). The case remained closed and V1 remained open for as long as there was a supply of T_2 in the chamber.

In order to draw a small volume of T_2 and its carrier He for an experiment, valves V2 and V3 were opened. The gas passed from the storage chamber (PC1) through stainless steel piping to a brass chamber (PC2; 250 ml internal volume). The PC2 was fitted with a 0-30 psi pressure gauge (G4; WIKA Instrument Corp., Lawrenceville, GA), and was controlled by its own inlet and outlet ports. This chamber contained a water vapor absorbent (anhydrous $CaSO_4$, Drierite®, WA Hammond Drierite Co., Xenia, OH). The gas entered from the bottom via a short length of copper pipe, and percolated

through the desiccant to remove contaminating tritiated water vapor. The gas then exited from the top of the chamber and passed through stainless steel piping to a second brass pressure chamber (PC3; 250 ml internal volume); this was the experimental chamber. When Valve VS1 was in the LP position, a low pressure (0 - 30 psi) gauge (G2; Pacific Scientific, Newport Beach, CA) read the pressure in the experimental chamber. Micrometering Valve V5 controlled gas entry through the system to the experimental chamber, and Valve V7 on the top of the chamber controlled the flow directly into it.

After introducing the T_2 into the experimental chamber, valves V2, V3, and V5, and the exit port from the desiccant chamber (PC2) were closed. Valve VS1 was switched to the HP position so that a high pressure (0 - 1000 psi) gauge (G3; US Gauge) could read the pressure in the experimental chamber (PC3).

A tank of He was connected to C2, and V10 was opened. With V5 and V7 open, the experimental chamber was compressed with He to 150 psig (10 atm) at a rate of $2 \text{ atm} \cdot \text{min}^{-1}$. Valve V10 was then closed. The whip from the H_2 tank, which was also connected to C2, was flushed with He. Valve V10 was reopened, and compression of the experimental chamber continued, using H_2 to a pressure of either 300 psig (10 atm H_2) or 885 psig (50 atm H_2).

At the end of an experiment, opening Valves V5, V6, and V7 allowed the PC3 chamber gases to be vented, controlling the rate with the micrometering Valve V5. Decompression rate was $0.4\text{-}0.5 \text{ atm} \cdot \text{min}^{-1}$. A stream of air, supplied by the building's laboratory gas air banks, was sent through a flow meter (FM1; Cole-Parmer Instrument Co., Chicago, IL). The gases from the experimental chamber (PC3) were vented through

another flow meter (FM2; Scott Specialty Gases, Plumsteadville, PA) into the stream of air at a volume ratio of 1/100. The combined gas stream passed through a pair of combustion traps linked in series. These combustion traps were glass vessels filled with palladium-coated molecular sieve material (Science Glass Inc., Miami, FL). The Pd catalyzed the production of water from H_2 and O_2 (Östlund and Mason, 1974) and the molecular sieve material absorbed all the water thus formed. The gas stream then flowed past a H_2 sensor (Sensidyne Inc., Clearwater, FL) before venting to the atmosphere. When decompression was completed, the chamber was flushed 3 times with He before opening.

TABLE 1

H₂ oxidation rate (mean \pm 1 SD) estimated from T₂ incorporation rate for mammalian tissue homogenates and dice (mean of 12 tissues), palladium beads immersed in water (6 samples) and suspensions of the bacterium *Alcaligenes eutrophus* (6 samples), following 1 h exposure to 8 mCi T₂ and either 10 or 50 atm H₂.

	10 atm H ₂ (moles H ₂ · g ⁻¹ · min ⁻¹)	50 atm H ₂ (moles H ₂ · g ⁻¹ · min ⁻¹)
Mammalian Tissues	1.1 x 10 ⁻⁸ (\pm 0.98)	4.8 x 10 ⁻⁸ (\pm 4.4)
Palladium	1.3 x 10 ⁻⁵ (\pm 0.41)	3.1 x 10 ⁻⁵ (\pm 1.9)
<i>A. eutrophus</i>	9.5 x 10 ⁻⁴ (\pm 1.5)	1.9 x 10 ⁻³ (\pm 0.45)

FIGURE LEGENDS

Figure 1. Simplified flow chart of tritium apparatus.

Figure 2. Technical diagram of tritium apparatus, showing (a) the three pressure chambers (PC), and the manifold including valves (V), check valves (symbolized as a circle and V), gauges (G), and flow meters (FM), and (b) the exhaust system including the thermistors, combustion traps and H₂ sensor.

Figure 3. Total tritium activity in samples of tissue homogenates (means of 3 samples from 1 organ \pm 1 SD), dice (means of 3 samples of 1 organ \pm 1 SD), or live cell cultures (means of 6 samples from 1 culture \pm 1 SD) from guinea pigs, rats or pigs following 1 h exposure to 8 mCi T₂ and 10 atm H₂. Also included is the total tritium activity in sterile, distilled water (mean of 7 samples \pm 1 SD) exposed to T₂ and H₂ under the same conditions.

Figure 4. Total tritium activity in samples of tissue homogenates (means of 3 samples from 1 organ \pm 1 SD), dice (means of 3 samples of 1 organ \pm 1 SD), or live cell cultures (means of 6 samples from 1 culture \pm 1 SD) from guinea pigs, rats or pigs following 1 h exposure to 8 mCi T₂ and 50 atm H₂. Also included is the total tritium activity in sterile, distilled water (mean of 7 samples \pm 1 SD) exposed to T₂ and H₂ under the same conditions.

Figure 5. Total tritium activity in all mammalian tissues studied (mean of 15 tissues \pm 1 SD), in sterile, distilled water (mean of 7 samples \pm 1 SD), and in 2 positive controls of Pd beads immersed in water (mean of 6 samples \pm 1 SD) and cultures of the hydrogen-metabolizing bacterium *Alcaligenes eutrophus* (mean of 6 samples \pm 1 SD), following 1 h exposure to 8 mCi T₂ and 10 atm H₂.

Figure 6. Total tritium activity in all mammalian tissues studied (mean of 15 tissues \pm 1 SD), in sterile, distilled water (mean of 7 samples \pm 1 SD), and in 2 positive controls of Pd beads immersed in water (mean of 6 samples \pm 1 SD) and cultures of the hydrogen-metabolizing bacterium *Alcaligenes eutrophus* (mean of 6 samples \pm 1 SD), following 1 h exposure to 8 mCi T₂ and 50 atm H₂.

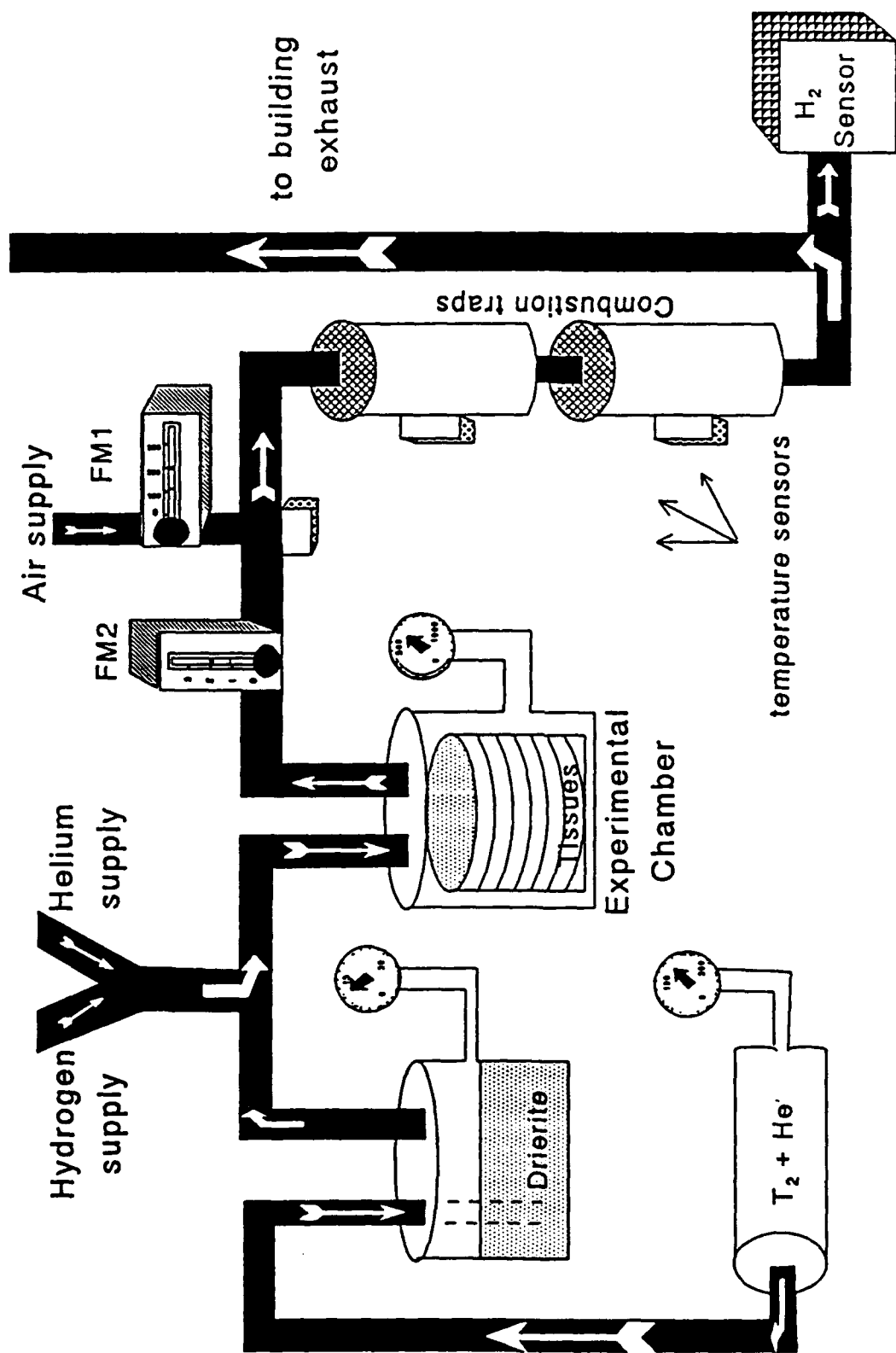


FIGURE 2a

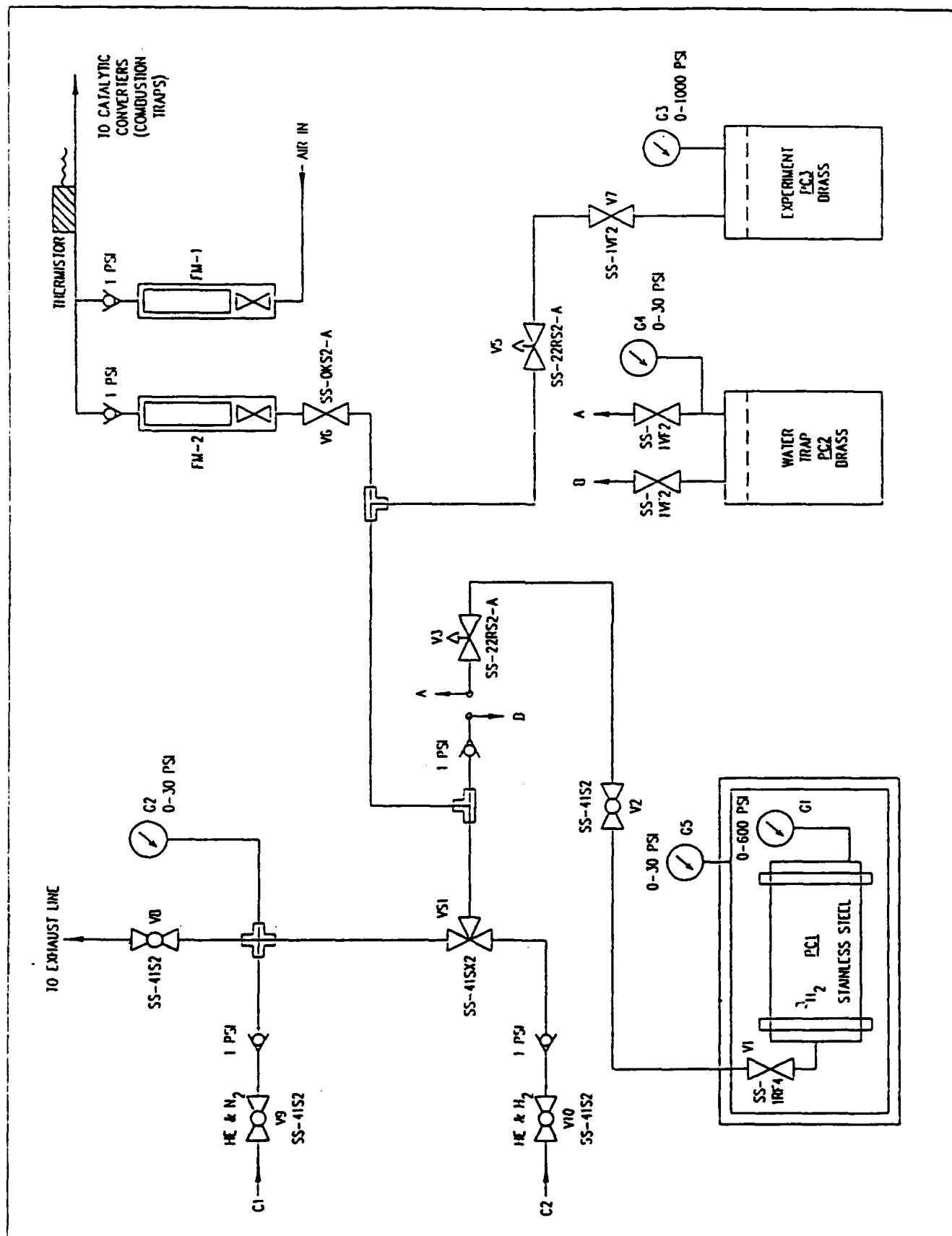


FIGURE 2b

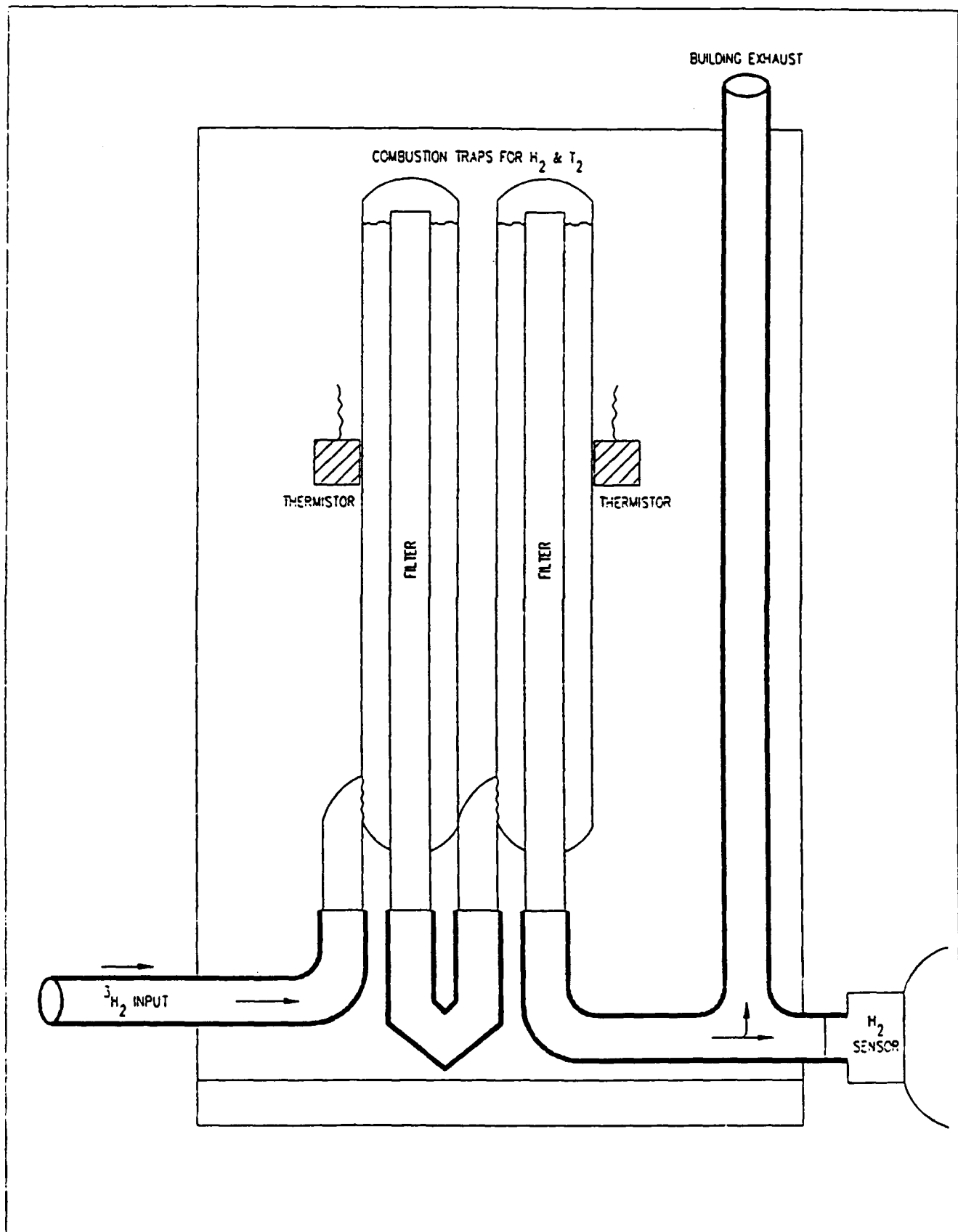


FIGURE 3

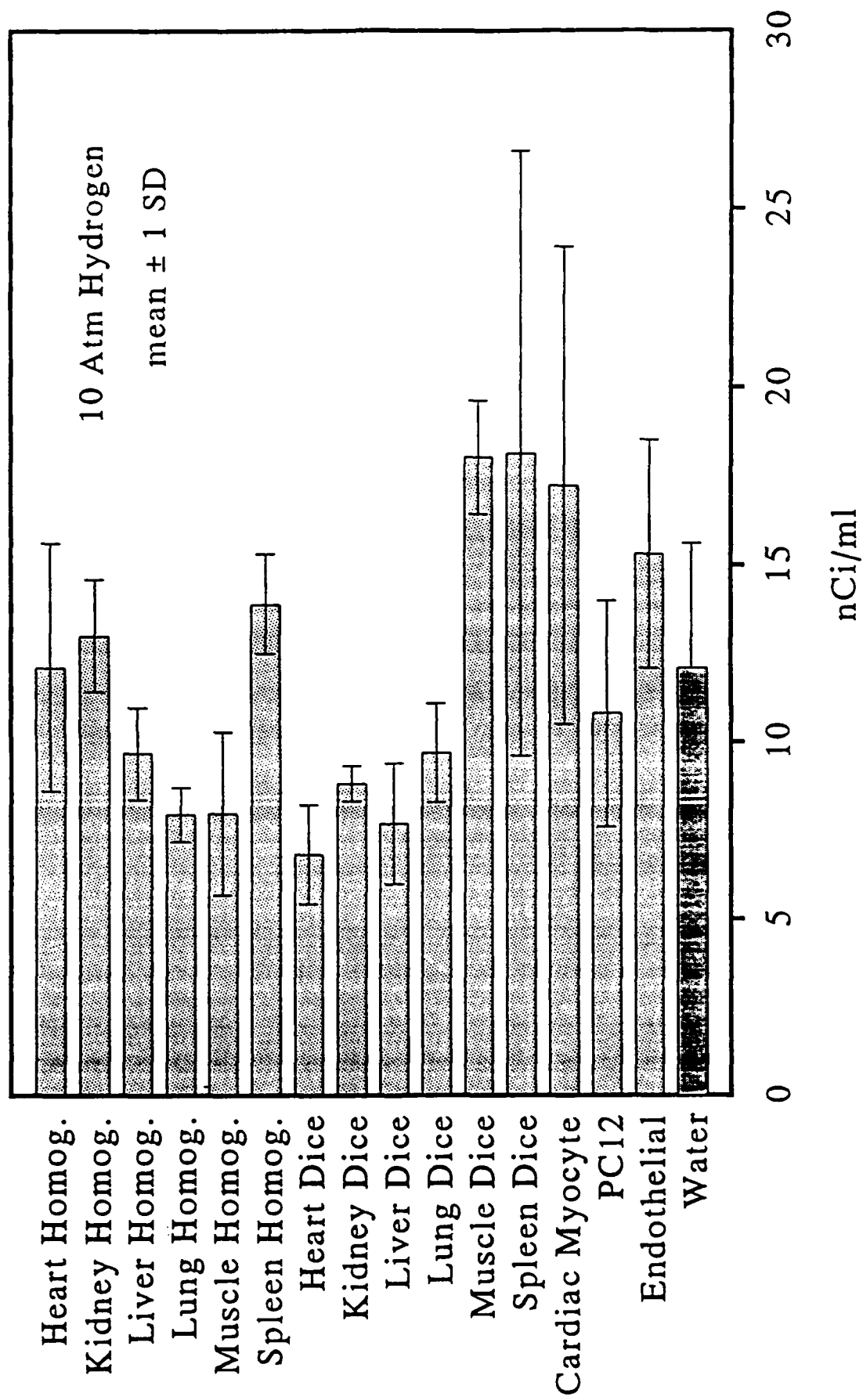


FIGURE 4

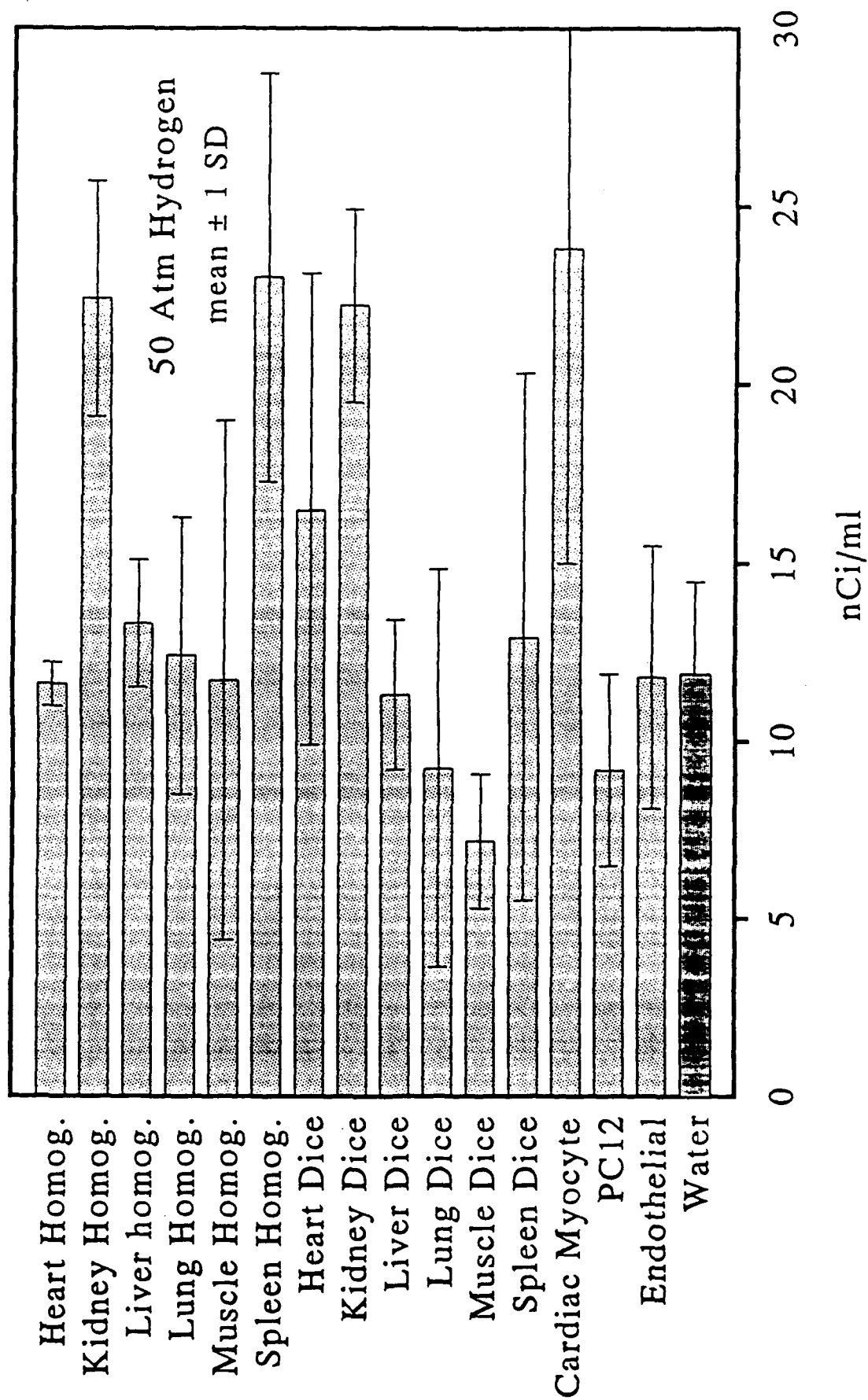


FIGURE 5

